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Binding of the RamR Repressor to Wild-Type and Mutated Promoters of the *ramA* Gene Involved in Efflux-Mediated Multidrug Resistance in *Salmonella enterica* Serovar Typhimurium

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The transcriptional activator RamA is involved in multidrug resistance (MDR) by increasing expression of the AcrAB-TolC RND-type efflux system in several pathogenic *Enterobacteriaceae*. In *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), *ramA* expression is negatively regulated at the local level by RamR, a transcriptional repressor of the TetR family. We here studied the DNA-binding activity of the RamR repressor with the *ramA* promoter (P_{ramA}). As determined by high-resolution footprinting, the 28-bp-long RamR binding site covers essential features of P_{ramA}, including the −10 conserved region, the transcriptional start site of *ramA*, and two 7-bp inverted repeats. Based on the RamR footprint and on electrophoretic mobility shift assays (EMSAs), we propose that RamR interacts with P_{ramA} as a dimer of dimers, in a fashion that is structurally similar to the QacR-DNA binding model. Surface plasmon resonance (SPR) measurements indicated that RamR has a 3-fold-lower affinity (K_D [equilibrium dissociation constant] = 191 nM) for the 2-bp-deleted P_{ramA} of an MDR *S.* Typhimurium clinical isolate than for the wild-type P_{ramA} (K_D = 66 nM). These results confirm the direct regulatory role of RamR in the repression of *ramA* transcription and precisely define how an alteration of its binding site can give rise to an MDR phenotype.

Multidrug resistance (MDR) achieved by intrinsic efflux systems is a major resistance mechanism used by bacteria to resist antimicrobial treatments, and it therefore represents a serious health problem worldwide. Most bacteria intrinsically possess several membrane transport systems which can decrease the intracellular concentration of toxic compounds, including antimicrobials of various classes and natural compounds present in animal hosts, such as bile salts (10, 20). The transcriptional regulation of those transport systems is achieved at two different levels: by local regulators encoded in the same gene clusters as efflux pumps and by global regulators encoded in other genomic regions and whose regulatory action can also affect functions other than MDR (10, 14, 15, 18). In several pathogens such as *Salmonella* spp., *Enterobacter aerogenes*, *Enterobacter cloacae*, and *Klebsiella pneumoniae*, the RamA global transcriptional activator, which belongs to the AraC/XylS family of regulatory proteins, participates in MDR by activating the expression of the AcrAB RND-type efflux pump (1, 2, 5, 8, 10, 19, 23). In *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), RamA has also been reported to negatively influence virulence (2, 13). We previously identified, directly upstream of *ramA*, the *ramR* gene coding for a protein of the TetR family of transcriptional repressors (1). Its role as a local repressor of *ramA* was determined by inactivation and complementation experiments (1). Various types of mutations in *ramR* or in the *ramR-ramA* intergenic region were identified in multidrug-resistant or quinolone-resistant strains of *S.* Typhimurium and other *S. enterica* serovars, which result in increased expression of *ramA* and increased efflux-mediated MDR (1, 9).

Members of the TetR family of transcriptional repressors control genes whose products are involved in various bacterial processes such as the biosynthesis of antibiotics, efflux-mediated resistance, and adaptation to osmotic stress or pathogenicity (17). They are two-domain proteins, with a highly conserved N-terminal DNA-binding domain comprising a helix-turn-helix (HTH) motif and a variable C-terminal ligand-binding regulatory domain. TetR-like proteins bind DNA in the control region of the genes that they regulate, thus blocking the initiation of their transcription (17, 20). Many of the TetR-like efflux regulators recognize and respond to molecules which are substrates of the efflux systems that they control (10, 18). This ligand binding induces a conformational change that abolishes the DNA binding and therefore the repression activity (16–18, 21). In the particular case of the RamR transcriptional repressor of *S.* Typhimurium, we previously predicted a putative DNA-binding site in the regulatory region of *ramA* located in the 288-bp *ramR-ramA* intergenic region (1). In the present study, we precisely define the DNA-binding site of RamR within the *ramA* promoter (P_{ramA}) and we propose an interaction model. We also show how a mutational alteration of P_{ramA} can compromise RamR binding and therefore lead to enhanced expression of the AcrAB-TolC MDR efflux system.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 1. MDR *S.* Typhimurium DT104 strains were isolated from cattle in Belgium (strain 543SA98)

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid name	Antibiotic resistance profile[a]	Relevant characteristic(s)	Purpose	Reference or source
Bacterial strains				
S. Typhimurium				
ATCC 14028s	Susceptible	Wild type	*ramR* cloning, EMSA, and qRT-PCR	6
14028sΔ*ramR*::*kan*	Susceptible	Laboratory *ramR*-deleted mutant	qRT-PCR	This study
543SA98	AMP, CHL, NAL, SPT, STR, SUL, TET	*ramA* overexpression due to a *ramR* mutation	RACE-PCR	1
BN10055	AMP, CHL, NAL, SPT, STR, SUL, TET	2-bp deletion at the RamR binding site	EMSA and qRT-PCR	1
E. coli BL21(DE3)pLysS	AMP, CHL	F^- *ompT* $hsdS_B(r_B^-\ m_B^-)$ *gal dcm* (DE3) pLysS	Overproduction of His_6-RamR	Promega
Plasmids				
pET15b	AMP	Cloning/expression vector	*ramR* cloning	Novagen
pET15bramR	AMP	Recombinant pET15b carrying the *S.* Typhimurium *ramR* gene	*ramR* expression	This study

[a] AMP, ampicillin; CHL, chloramphenicol; NAL, nalidixic acid; SPT, spectinomycin; STR, streptomycin; SUL, sulfonamide; TET, tetracycline.

and France (strain BN10055). Isolate 543SA98 has a frameshift mutation in *ramR* resulting in an overexpression of *ramA*, and isolate BN10055 has a 2-bp deletion in the putative RamR DNA-binding site located upstream of *ramA* (1). Mutant 14028sΔ*ramR*::*kan* derived from the susceptible strain 14028s was constructed as previously described (1). Except where indicated, the bacterial strains were grown overnight at 37°C in Luria-Bertani (LB) broth. The pET15b vector (Novagen, Merck KGaA, Darmstadt, Germany) was used to clone and express the *ramR* gene. *Escherichia coli* BL21(DE3)pLysS was used as the host strain to overproduce the N-terminally hexahistidine-tagged RamR protein (His_6-RamR).

Identification of the *ramA* transcriptional start site. RNA was extracted from a culture of the MDR *S.* Typhimurium strain 543SA98 grown until it reached an optical density at 600 nm (OD_{600}) of 0.5 using the NucleoSpin RNAII kit (Macherey-Nagel, Hoerdt, France). Specific primers (Sigma-Aldrich, Saint-Quentin Fallavier, France) SP1, SP2, and SP3 (Table 2) were designed to determine the 5′ end of the *ramA* transcript by using the second-generation 5′/3′ RACE (rapid amplification of cDNA ends) kit (Roche Diagnostics, Bâle, Switzerland) according to the manufacturer's instructions. The resulting PCR product was sequenced by Cogenics (Meylan, France).

Overproduction and purification of RamR. Chromosomal DNA of the *S.* Typhimurium 14028s strain was prepared with a QIAamp DNA minikit (Qiagen, Courtaboeuf, France). The *ramR* gene was amplified by PCR using Dynazyme polymerase (Ozyme, Montigny-Le-Bretonneux, France) and primers ramRXhoI and ramRNdeI (Sigma-Aldrich; Table 2). The PCR product (595 bp) was cut by XhoI and NdeI (Promega, Madison, WI) and cloned into the corresponding cloning site of pET15b. The nucleotide sequence of the resulting pET15bramR recombinant plasmid was confirmed by sequencing. The *E. coli* BL21(DE3)pLysS strain was transformed with pET15bramR and grown at 20°C in 2YT broth (tryptone, 16 g/liter; yeast extract, 10 g/liter; NaCl, 5 g/liter) containing ampicillin (50 mg/liter) (Fluka Sigma-Aldrich, Saint-Quentin Fallavier, France) and chloramphenicol (30 mg/liter) (Fluka Sigma-Aldrich). At an OD_{600} of 0.5, the production of recombinant protein was induced by the addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside (Calbiochem, Merck KGaA). Cultures were incubated for 16 h, and bacterial cells were then disrupted by three freezing and thawing cycles in the presence of lysozyme. The soluble protein His_6-RamR was purified using Talon metal affinity resin (Clontech Laboratories, Mountain View, CA) with a 20 mM Na_2HPO_4, 150 mM NaCl, 200 mM imidazole buffer and then by gel filtration on a Superdex S75 column (Pharmacia, GE Healthcare, Waukesha, WI) with a 10 mM HEPES, 150 mM NaCl, 5 mM dithiothreitol, 10% (wt/vol) glycerol buffer. The eluates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to check the purity of the His_6-RamR protein.

EMSAs. A fragment of the *ramR-ramA* intergenic region including the putative binding site of RamR was amplified by PCR using the Go *Taq* DNA polymerase (Promega) and primers interam3 and interam4 (Sigma-Aldrich; Table 2). The amplicons were 97 bp in length for the *S.* Typhimurium 14028s strain and 95 bp for the *S.* Typhimurium BN10055 strain.

TABLE 2 Primers used in this study

Primer purpose and name	Oligonucleotide sequence (5′ to 3′)
RACE-PCR	
SP1	CAGTTTTAGCTTCCGTTCAC
SP2	CTCACGCGTCGACATCAATCCACTCGACAATCG
SP3	CTCACGCGTCGACTCATCGTGCTCTCCCCTCTA
Cloning	
ramRXhoI	CTCCTCGAGACCGTCCATTATTGCTCCTC
ramRNdeI	CTCCATATGGTGTAGTGGCTCGTCCGAAG
EMSA	
interam3	ACCTTGACGGCGTATCTTTG
interam4	ATGGCCTGCAATATGCTTTT
gyrB4	CTTGTCCGGGTTGTACTCGT
gyrB5	GCTTCGACAAGATGCTTTCG
qRT-PCR	
gmk-f	TTGGCAGGGAGGCGTTT
gmk-r	GCGCGAAGTGCCGTAGTAAT
gyrB-f	TCTCCTCACAGACCAAAGATAAGCT
gyrB-r	CGCTCAGCAGTTCGTTCATC
rrs-f	CCAGCAGCCGCGGTAAT
rrs-r	TTTACGCCCAGTAATTCCGATT
acrB-f	TCGTGTTCCTGGTGATGTACCT
acrB-r	AACCGCAATAGTCGGAATCAA
ramA-f	GCGTGAACGGAAGCTAAAAC
ramA-r	GGCCATGCTTTTCTTTACGA
ramR-f	TAACGCAGGTGTTGCAGAAG
ramR-r	TGGTTCAGACCCCAACTGAT
tolC-f	GCCCGTGCGCAATATGAT
tolC-r	CCGCGTTATCCAGGTTGTTG

A control 92-bp amplicon of the *gyrB* gene was amplified from the *S.* Typhimurium 14028s strain using primers gyrB4 and gyrB5. The PCR products were purified using the NucleoSpin Extract II kit (Macherey-Nagel) and were digoxigenin (DIG) labeled with the DIG gel shift kit (Roche Diagnostics). The electrophoretic mobility shift assay (EMSA) reaction mixtures had a final volume of 11 μl and contained 15.5 fmol of labeled DNA and 0, 5, 10, or 15 pmol of the purified His_6-RamR protein in the binding buffer (36 mM HEPES, 240 mM KCl, pH 7.6). Competition assays were done under the same conditions with 15.5 fmol of the labeled *ramR-ramA* 97-bp DNA amplicon and various quantities of unlabeled DNA, in the presence of 10 pmol of His_6-RamR protein. After 15 min at room temperature, the samples were loaded onto 6% nondenaturing acrylamide gels, electrophoresed at 4°C in 0.5× Tris-buffered EDTA (TBE) buffer, and transferred onto a nylon membrane. The bands revealed by the DIG gel shift kit were visualized with the ChemiSmart 5000 unit and analyzed with the ChemiCapt 50001 software (Vilber Lourmat, Marne-la-Vallée, France).

High-resolution HRF. Hydroxyl radical footprinting (HRF), rather than DNase I footprinting, was chosen because it is not limited by the steric hindrance associated with the DNase I and DNA-binding proteins because of the small size of the diffusing chemical nuclease (hydroxyl radicals). It can therefore provide high-resolution footprinting of DNA-protein complexes and structural detail for them (7). The two complementary oligonucleotides corresponding to the 97-bp fragment of the *ramR-ramA* intergenic region were synthesized (Sigma-Aldrich) and separately 5′ labeled with [γ-^{32}P]ATP (Perkin-Elmer, Villebon-sur-Yvette, France) at 30 μCi per 10-μl reaction mixture using the T4 polynucleotide kinase (New England BioLabs, Ipswich, MA). Each labeled oligonucleotide was hybridized with its unlabeled complementary oligonucleotide. For binding assays, 100 nM purified radiolabeled DNA fragments were incubated for 15 min at room temperature with 0 or 32 μM His_6-RamR protein in 20 mM HEPES-NaOH, 240 mM NaCl (pH 7.6) buffer. Hydroxyl radical attacks were processed as previously described by Tullius and Dombroski (22) and adapted by Castaing et al. (3). Briefly, 10 μl of binding mix was incubated for 2 min at room temperature with 3 μl of fresh and cooled solution containing 0.1% (vol/vol) H_2O_2, 6.7 mM ascorbate, and 0.1 mM $[Fe(EDTA)]^{2-}$. Reactions were quenched by addition of 1.8 μl of stop solution containing 80 mM thiourea and 13 mM EDTA. Maxam-Gilbert chemical sequencing reactions were also performed (11). All samples were analyzed by electrophoresis on a denaturing 7% polyacrylamide gel. Quantification of radioactive signals was performed with the Storm apparatus and the ImageQuant software (Amersham Biosciences, GE Healthcare). The DNA footprints obtained were manually fitted to that deduced from the crystal structure of a complex formed between QacR, a member of the TetR family, and its operator site (Protein Data Bank [PDB] file 1JT0) (20).

Measurement of RamR interaction with the *ramA* operator by SPR. Surface plasmon resonance (SPR) experiments were performed using a Biacore T100 biosensor instrument (GE Healthcare). The 5′-biotinylated wild-type 97-bp and mutated 95-bp fragments of the *ramR-ramA* intergenic region, as well as the 92-bp control fragment of the *gyrB* gene, were immobilized to a level of 300 to 450 resonance units (RU) onto a neutravidin-coated CM5 sensor chip (GE Healthcare). Binding analyses were carried out at 25°C and at a flow rate of 30 μl/min. The His_6-RamR purified protein was diluted in the running buffer (10 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.05% [vol/vol] Tween 20, and 5 g/liter bovine serum albumin [BSA], pH 7.4) and injected over the sensor surface in 2 replicates for 5 min. Dissociation was recorded for 5 min. Regeneration of the surfaces was performed with 10 mM Tris, 2 M NaCl for 1 min followed by washes for 5 min. Binding curves were corrected for nonspecific background by subtracting the curves obtained with the control fragment and the running buffer alone. The calculations of kinetic or affinity constants were done with the Biacore T100 evaluation software (version 2.02) using two models, the classical single-interaction (1:1) Langmuir model or the conformational-change model. The latter is a two-state reaction model based on the formation of a complex between the analyte and the immobilized ligand followed by a conformational change stabilizing this complex. Results were evaluated with the chi-square test.

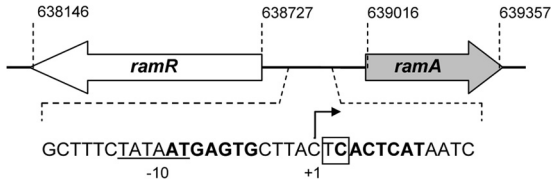


FIG 1 Genetic organization of the *ram* locus, showing the putative RamR binding site in the P_{ramA} region. The nucleotide positions are those of the *S.* Typhimurium strain LT2 genome sequence (GenBank accession number NC_003197). The −10 box is underlined, and the transcriptional start site (+1) is indicated by an arrow. Bold letters indicate inverted repeats. The 2 bp (TC) which are deleted in strain BN10055 are indicated in a box.

Gene expression analysis by qRT-PCR. Bacteria were grown until mid-log phase (OD_{600} of 0.6) and harvested by centrifugation. Pelleted cultures were stabilized with RNAprotect bacterial reagent (Qiagen) and stored at −80°C until use. Total RNA was extracted using the RNeasy minikit (Qiagen) according to the manufacturer's recommendations. Removal of residual genomic DNA was performed using the Turbo DNA-free kit (Ambion) and checked by negative PCR amplification of a chromosomal sequence. RNA integrity was checked by electrophoresis in a 1% agarose gel. Total RNAs were reverse transcribed using random hexamers and the Superscript III first-strand synthesis system (Applied Biosystems). Primers used for quantitative reverse transcription-PCR (qRT-PCR) are listed in Table 2. Cycling conditions were as follows: 95°C for 5 min followed by 40 cycles of 95°C for 10 s and 60°C for 15 s. After each run, amplification specificity and absence of primer dimer formation were checked with a dissociation curve acquired by heating the PCR products from 60 to 95°C. Relative quantities of transcripts were determined using the standard curve method and normalized against the geometric mean of three reference genes (*gmk*, *gyrB*, and *rrs*). Relative expression of each gene of interest (*acrB*, *ramA*, *ramR*, and *tolC*) was calculated as the average of three independent RNA samples. A two-tailed Student *t* test was used to assess significance, using a *P* value of <0.05 as a cutoff.

RESULTS AND DISCUSSION

RamR binding to P_{ramA}. To experimentally identify P_{ramA}, we determined its transcriptional start site. RACE-PCR assays showed that it consists of a C residue located 163 bp upstream of the predicted translational start site of RamA and corresponding to nucleotide position 638853 of the *S.* Typhimurium strain LT2 genome (GenBank accession number NC_003197) (12). As expected, the C(+1) residue is located 10 bp downstream of the −10 TATAAT box and is part of the 6-bp linker that separates two 7-bp inverted repeats (IRs), which were previously suggested to be part of the RamR binding site (Fig. 1) (1).

In order to study the interaction of RamR with P_{ramA}, we produced RamR in an *E. coli* host strain, as a recombinant His_6-RamR protein with a theoretical molecular mass of 24,304 Da (versus 21,786 Da for the 193-amino-acid-long predicted native protein). The protein was purified to homogeneity in its homodimeric form, according to the gel filtration retention time of the purified protein. We conducted EMSA with a 97-bp DNA fragment containing the putative RamR binding site in the presence or absence of His_6-RamR (Fig. 2, lanes 1 to 4). Addition of increasing amounts of His_6-RamR to the reaction mixture resulted in a decreased mobility of the 97-bp DNA fragment. A single retardation band was observed for the lowest tested protein input (5 pmol), whereas two retardation bands were observed for higher inputs

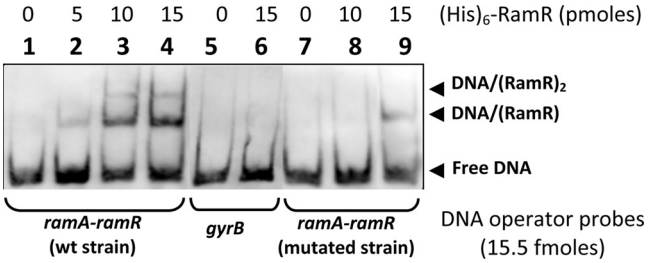


FIG 2 Electrophoretic mobility shift analysis of RamR interaction with the *ramR-ramA* intergenic region including P_{ramA}. Various amounts of the His_6-RamR protein were incubated with the 97-bp DNA fragment containing the putative binding site of RamR amplified from the *S.* Typhimurium 14028s strain (lanes 1 to 4) or with the corresponding 2-bp-deleted DNA fragment amplified from the *S.* Typhimurium BN10055 strain (lanes 7 to 9). Lanes 5 and 6 show, as a negative control, 0 and 15 pmol, respectively, of the His_6-RamR protein incubated with a 92-bp *gyrB* DNA fragment. wt, wild type.

(10 and 15 pmol). The intensity of these retardation bands increased with RamR concentration. These results indicate that the formation of DNA/RamR complexes can occur in a 1:1 or in a 1:2 molar ratio. No band shift was observed with a negative-control *gyrB* fragment in the presence of His_6-RamR (Fig. 2, lanes 5 and 6). Furthermore, the DNA/RamR complexes were readily competed in the presence of a 4-fold or more excess of the unlabeled DNA probe, which confirmed the specificity of the binding (data not shown).

To better decipher at the molecular level the interaction of RamR with P_{ramA}, the 97-bp DNA fragment described above was physically mapped using high-resolution hydroxyl radical footprinting (HRF) (7). Footprinting experiments were performed on both the top strand (i.e., the *ramA* coding strand) and the bottom strand of the DNA fragment. The resulting footprint was symmetric and extended over 28 bp comprising the P_{ramA} predicted −10 region, the IR sequences, the 6 extra base pairs of the IR linker, and additional base pairs on the external sides of the IR (Fig. 3A and B). The RamR footprints were not completely identical on the two strands, but they displayed similar bipartite profiles (from 5′ to 3′, CTATAATGA and CTTACTCAC on the top strand and ATTAC GAGT and TAAGCACTCATT on the bottom strand). The overlapping of the −10 region and of the *ramA* transcriptional start by the DNA footprint of RamR unambiguously supports the direct repression of *ramA* by RamR.

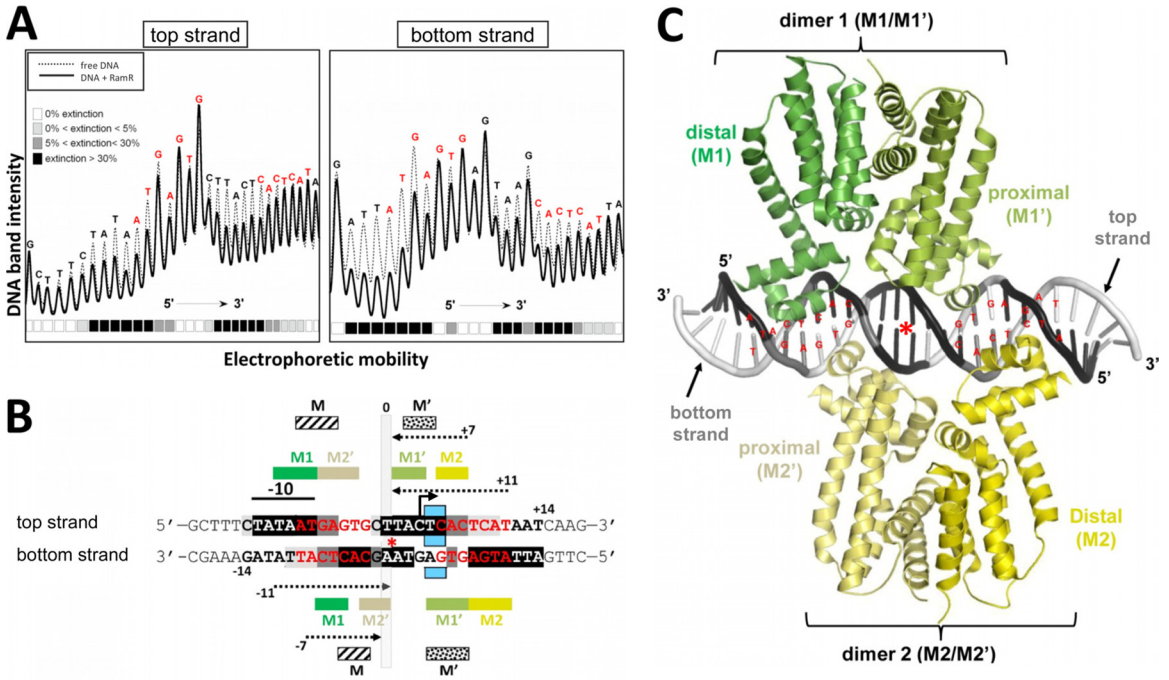


FIG 3 Hydroxyl radical footprinting of the *ramR-ramA* intergenic region with RamR. (A) Electrophoretic profiles of hydroxyl radical attack obtained on DNA top strand and bottom strand in the absence (dotted lines) or in the presence (thick lines) of RamR. The nucleotide sequence is noted at the top of each panel, with IR sequences indicated in red. Boxes at the bottom of each panel correspond to the nucleotides of the DNA fragments, and their gray intensity indicates the signal extinction in the presence of RamR, according to the code indicated in the left panel. (B) Schematic representation of the RamR footprint and comparison with the TetR and QacR DNA-binding sites. The nucleotide sequence recognized by RamR is indicated by bold letters and delimited by the numbers −14/+14 from the 2-fold symmetric axis (indicated by a red asterisk). The gray intensity of the base background follows the code defined in panel A. The IR sequences are shown in red. The −10 region of P_{ramA} is indicated, and the transcriptional start site of *ramA* is shown with a black arrow. The 2-bp deletion which alters the RamR DNA binding in strain BN10055 is indicated by a blue box. The vertical gray barrel corresponds to the extra base pair "0" of the 15-bp palindromic TetR operator. The positions of the IRs (−7, 0, +7) present in the TetR DNA-binding site are indicated by black dotted arrows. Black hatched and dotted boxes indicate the physical protein-DNA interactions identified in the crystal structure of the TetR-DNA complex (PDB file 1QPI), with M and M′ designating the protomers of the TetR homodimer. The positions of the IRs (−11, +11) present in the QacR DNA-binding site are also indicated by black dotted arrows. Green boxes indicate the physical QacR-DNA interactions, with M1/M1′ and M2/M2′ designating each protomer of homodimer 1 and homodimer 2, respectively. (C) Ribbon representation of the QacR-DNA complex, showing the RamR footprint reported on the DNA sugar-phosphate backbone. The gray scale used for the DNA backbone follows the code defined in panel A. The 2-fold symmetric axis of the RamR footprint (indicated by a red asterisk) is superimposed on that of the crystal structure of the QacR-DNA complex (20). The structural representation was generated by PyMOL (PyMOL Molecular Graphics System; http://www.pymol.org).

The results of the EMSAs, as well as the large extent of the RamR footprint, support the idea that two RamR homodimers actually bind to the DNA target site. This hypothesis is further supported by literature data. The footprinting patterns that we obtained for RamR are clearly different from the one predicted by the crystal structure of the single homodimer $(TetR)_2$ of Gram-negative bacteria, which binds to its 15-bp operator in the *tetA* promoter region (Fig. 3B) (16). Instead, the RamR footprints better match the DNA-protein contacts extracted from the crystal structure of the two $(QacR)_2$ homodimers, which bind cooperatively to the *qacA* 28-bp operator of *Staphylococcus aureus* (Fig. 3B and C) (20). The RamR binding pattern is also similar to the *Escherichia coli* AcrR repressor, which binds as a dimer of dimers to a 24-bp sequence of the *acrAB* operator, including two 10-bp IR sequences separated by a 4-bp linker (21).

A 2-bp deletion in P_{ramA} alters the RamR DNA binding. We previously described the *S.* Typhimurium DT104 BN10055 MDR isolate, which has a 2-bp deletion at the junction between the IR linker and one of the IR sequences of the P_{ramA} region (Fig. 1) (1). This isolate produces the AcrA protein at an increased level (about 4-fold more than a strain with a wild-type efflux activity). Furthermore, the inactivation of the *ramA* gene of this isolate resulted in a 4-fold decrease in the MICs of antibiotic known to be effluxed by the AcrAB-TolC system. These results suggested that the 2-bp deletion played a key role in the MDR phenotype of this isolate, by compromising the repression exerted by RamR on the *ramA* promoter and therefore activating the expression of the AcrAB-TolC efflux system. This hypothesis is here further supported by the increased level of transcription of the *ramA*, *acrB*, and *tolC* genes in the BN10055 isolate in comparison to the wild-type *S. enterica* serovar Typhimurium reference strain 14028s (Fig. 4A). Similar increases of transcription level were obtained for these genes in a *ramR*-deleted mutant strain in comparison with its 14028s parental strain (Fig. 4B). Whereas various mutations in the *ramR* gene were reported for MDR *Salmonella* mutants (1, 9), alteration of the RamR binding site was reported only once in a *Salmonella enterica* serovar Paratyphi B spontaneous mutant selected on ciprofloxacin (9). This prompted us to compare the molecular interactions of RamR with a wild-type P_{ramA} and with the mutated P_{ramA} of the BN10055 isolate.

First, we performed EMSAs with the 95-bp DNA fragment carrying the 2-bp-deleted P_{ramA} of the BN10055 clinical strain (Fig. 2, lanes 7 to 9). Results showed an altered binding of the His_6-RamR protein to this mutated P_{ramA}, compared to that observed with the wild-type P_{ramA}. In addition, even at the highest RamR input tested, only one homodimer was able to bind DNA, as shown by the single retardation band observed (Fig. 2, lane 9). Thus, this apparent loss of affinity may be associated with the inability of RamR to bind the mutated P_{ramA} as a dimer of homodimers.

Second, we conducted SPR experiments using the 97-bp and the 95-bp DNA fragments to quantify the RamR interactions with the wild type and with the 2-bp-deleted P_{ramA}, respectively. Over a 3 to 100 nM range of concentrations of the His_6-RamR protein, SPR sensorgrams showed an increasing binding to the immobilized DNA fragment of the wild-type P_{ramA} (Fig. 5A). The best fitting was obtained with the conformational-change model rather than with the 1:1 Langmuir model. This corroborates that two RamR homodimers bind to the wild-type P_{ramA} and suggests that the binding of a first homodimer allows, by inducing a conformational change, the binding of a second homodimer. An average K_D value of 65.8 ± 0.2 nM was calculated from the kinetic constants indicated in Fig. 5B. This K_D value in the nanomolar range indicates a high affinity (defined as $1/K_D$) of RamR for its DNA-binding site, as observed for other members of the TetR family. For comparison, the affinity of RamR is higher than that of CmeR (K_D = 88 nM) but lower than that of AcrR (K_D = 20.2 nM) or QacR (K_D = 5.7 nM) for their respective binding sites (21). SPR sensorgrams also showed a dose-dependent binding of RamR to the 2-bp-deleted P_{ramA} (Fig. 5A). The calculated K_D for this interaction was 191.3 ± 0.07 nM, which indicated a 3-fold-lower affinity than that obtained with the wild-type P_{ramA}. This K_D value was obtained using the 1:1 Langmuir model, which gave the best fitting. This is in agreement with the EMSA results, which indicated the binding of a single RamR homodimer. Binding experiments (EMSA and SPR) both indicated that the 2-bp deletion observed in the P_{ramA} of the BN10055 strain affects the binding affinity of RamR and its ability to bind DNA as a dimer of dimers.

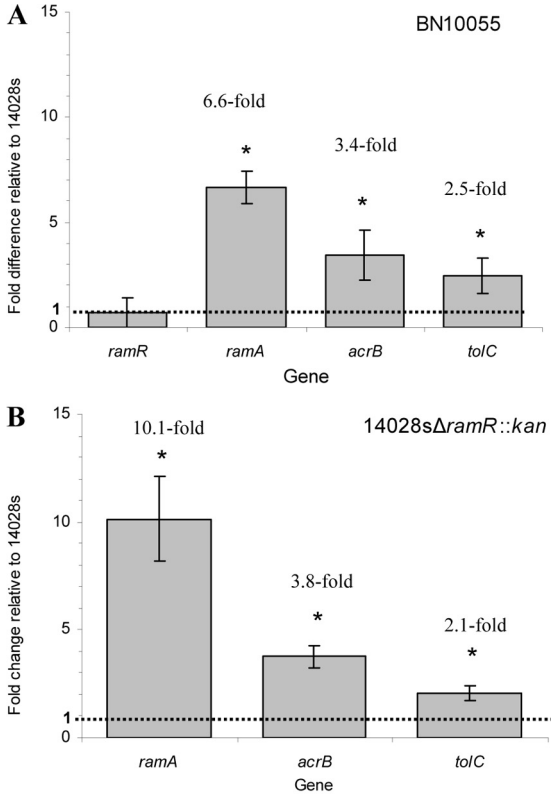
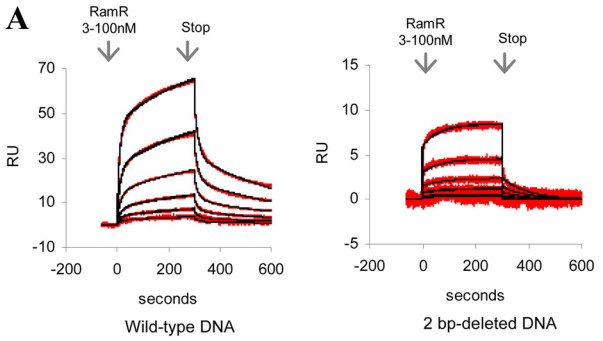


FIG 4 qRT-PCR analysis of *ramR*, *ramA*, *acrB*, and *tolC* expression. (A) Strain BN10055 (2 bp deleted in the P*ramA* region); (B) strain 14028sΔ*ramR*::*kan*. Asterisks show significant differences ($P < 0.05$). The dotted line indicates a fold change difference of 1 (i.e., no change).

The model that we propose for the binding of RamR to its target DNA postulates a direct contact between a RamR homodimer (monomer M2, Fig. 3B) and the 2 nucleotides of the top strand which are deleted in the mutated P_{ramA} of the BN10055 isolate. Based on all experimental results and on the three-dimensional (3D) model of QacR bound to DNA (Fig. 3C), we also propose a model for the altered binding of RamR to the mu-



B

Constant	Wild-type DNA fragment Conformational change model	2 bp-deleted DNA fragment Single interaction model
ka1 ($M^{-1}s^{-1}$)	$(3.30 \pm 0.01) \times 10^{5}$	$(5.40 \pm 0.12) \times 10^{4}$
kd1 (s^{-1})	$(6.10 \pm 0.01) \times 10^{-2}$	$(1.00 \pm 0.005) \times 10^{-2}$
ka2 ($M^{-1}s^{-1}$)	$(4.00 \pm 0.01) \times 10^{-3}$	
kd2 (s^{-1})	$(2.20 \pm 0.004) \times 10^{-3}$	
K_D (nM)	65.8	191.3
Chi2	0.2	0.07

FIG 5 Surface plasmon resonance analysis of RamR interaction with the *ramR-ramA* intergenic region including P*ramA*. (A) Biotinylated DNA fragments were immobilized on a neutravidin-coated sensor chip. Sensorgrams for 3 to 100 nM His_6-RamR showing its binding to the 97-bp DNA fragment of the wild-type *S.* Typhimurium 14028s strain and to the 95-bp (2-bp-deleted) DNA fragment of the BN10055 strain. The red lines represent experimental double-subtracted binding curves in duplicate. The black lines represent the fitted curves obtained with a conformational change model (wild-type DNA) and with a single-interaction model (2-bp-deleted DNA). (B) Kinetic and affinity constants obtained from SPR experiments. Association constant, K_a; dissociation constant, K_d; equilibrium dissociation constant, K_D.

tated P_{ramA}. In this model, the RamR homodimer 2 (i.e., M2/M2′) is unable to correctly bind the mutated operator, whereas homodimer 1 (M1/M1′) is still able to bind but, however, with a decreased affinity. Figure 6 presents RamR binding to the top strand, whose footprint best fits that predicted from the QacR/DNA complex. As an alternative, we also propose that a putative partial binding of the homodimer 2 by its M2 monomer may compromise the binding of homodimer 1.

Concluding remarks. In summary, we defined accurately the RamR binding site in the *ramA* regulatory region. The biochemical data provided precisely the molecular basis for the repression of *ramA* transcription by the RamR local repressor. They also demonstrate how an MDR phenotype is achieved in the particular BN10055 clinical isolate, whose 2-bp deletion at the RamR binding site compromises the *ramA* repression and enhances the expression of the *acrAB* and *tolC* efflux genes. Another recent study conducted in *Salmonella enterica* serovar Typhi showed that RamR also interacts with a non-protein-coding RNA, which suggests that the transcriptional regulation of the *ram* locus is actually more complex than a single DNA-protein interaction and warrants further investigation on the interactive roles played by each regulatory component (4). This appears also of particular importance since the *ram* locus has recently been shown to be also involved in the regulation of the expression of virulence genes in *S.* Typhimurium, such as those encoded by the pathogenicity island SPI-1 (2).

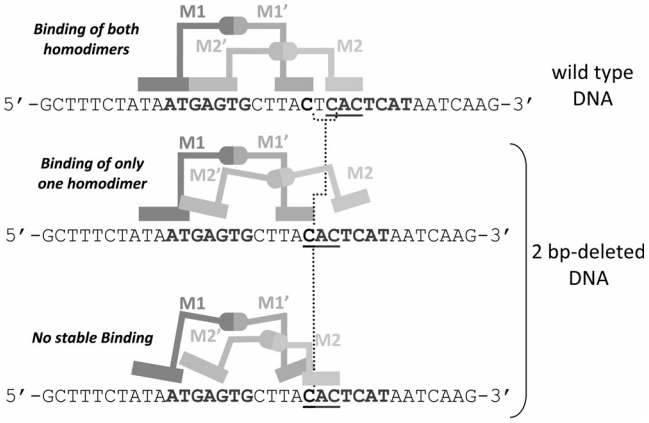


FIG 6 Comparative models for the DNA binding of RamR to the wild-type or mutated P*ramA* region. Two alternative models are proposed for the altered binding of the RamR dimer of dimers to the mutated (2-bp-deleted) *ramA* operator of strain BN10055.

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